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Glucose transport in the extremely thermoacidophilic *Sulfolobus solfataricus*

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Abstract. The archaeon *Sulfolobus solfataricus* grows optimally at a temperature of 80°C and a pH of 2.5-3.5 on carbon sources such as yeast extracts, tryptone and various sugars. Cells rapidly accumulate glucose. This transport activity involves a membrane-bound glucose-binding protein that interacts with its substrate with very high affinity (K_d of 0.43 μM) and retains high glucose affinity at very low pH values (up to pH 0.6). The binding protein was extracted with detergent, and purified to homogeneity as a 65-kDa glycoprotein. The gene coding for the binding protein was identified in the *S. solfataricus* P2 genome. Sequence analysis suggests the protein to be anchored to the membrane via an amino terminal transmembrane segment. Neighbouring genes encode two membrane proteins and an ATP-binding subunit that are transcribed in the reverse direction, whereas a homologous gene cluster in *Pyrococcus horikoshii* OT3 was found to be organised in an operon. These data indicate that *S. solfataricus* utilizes a binding-protein-dependent ATP-binding cassette (ABC) transporter for the uptake of glucose.

1. Introduction

Sulfolobus solfataricus is able to live in very acidic (pH 2-5) and very hot (up to 87°C) environments. It is a member of the third kingdom of life, the Archaea. Most members of this group exhibit the ability to survive extreme environmental conditions such as very high or low temperature, very acidic or alkaliphilic pH values, high salinity or high pressure. Woese et al. [26] postulated the Archaea as a distinct kingdom in 1990 based on studies on 16sRNAs. The analysis of the genome sequences of many different Archaea (*Methanococcus janaschii*, *Methanobacterium thermoautotrophicum*, *Archaeoglobus fulgidus*, *Pyrococcus furiosus*, *Sulfolobus solfataricus*) emphasizes their genetically different position. Two third of the genes found in these Archaea do not have homologues in Bacteria and Eucarya. The membranes of Archaea contain ether lipids in which two phytanyl chains are ether-linked to glycerol or another polyalcohol, nonitol. The lipid chains are composed of isoprene subunits, which contain methyl sidegroups at every fourth carbon atom of the chain backbone. Membrane spanning (bolaform amphiphilic) tetraether lipids are found in extreme thermophiles and acidophiles [6] (Fig.1). These lipids have a C_{40} isoprenoid phytanoyl chain that spans the entire membrane. Freeze-fracturing of liposomes made from tetraether lipids reveals that cleavage between two leaflets as seen

with diether or diester lipids does not occur [7]. Liposomes made of *S. acidocaldarius* derived tetraether lipids are characterized by a high stability and a very low proton permeability [9]. These liposomes were already used for the functional reconstitution of several membrane proteins such as cytochrome-c-oxidase of *B. stearothermophilus* and beaf heart, bacteriorhodopsin of *H. halobium* [8].

The archaeon *Sulfolobus solfataricus* grows optimally at a temperature of 80°C and a pH of 2 - 3.5 on carbon sources such as yeast extracts, tryptone and various sugars. As an acidophile *Sulfolobus* faces the problem of a high ΔpH, acid outside. It has been shown that the cytoplasm has a pH value near to neutral [19] so that the pH gradient across the membrane can be maintained only when the proton permeability of the membrane is very low. Extensive studies with liposomes from *Sulfolobus acidocaldarius* lipids revealed that the proton permeability of these membranes at 80°C is the same as for *E. coli* membranes at 20°C [8, 23]. The high ΔpH results in a large proton motive force (PMF), which is slightly reduced by a reversed Δψ (inside positive), but with a value of about -200 mV it is still high [18]. The reversed Δψ seems not to have any consequences for the orientation of the transmembrane segments of membrane proteins in *Sulfolobus* [24]. In neutrophiles membrane proteins carry a net positive charge in their intracellular loops (positive inside rule [25]). Also in acidophiles, the positive inside rule applies, and therefore the Δψ can not be a determinant for membrane protein topology as suggested earlier [3].

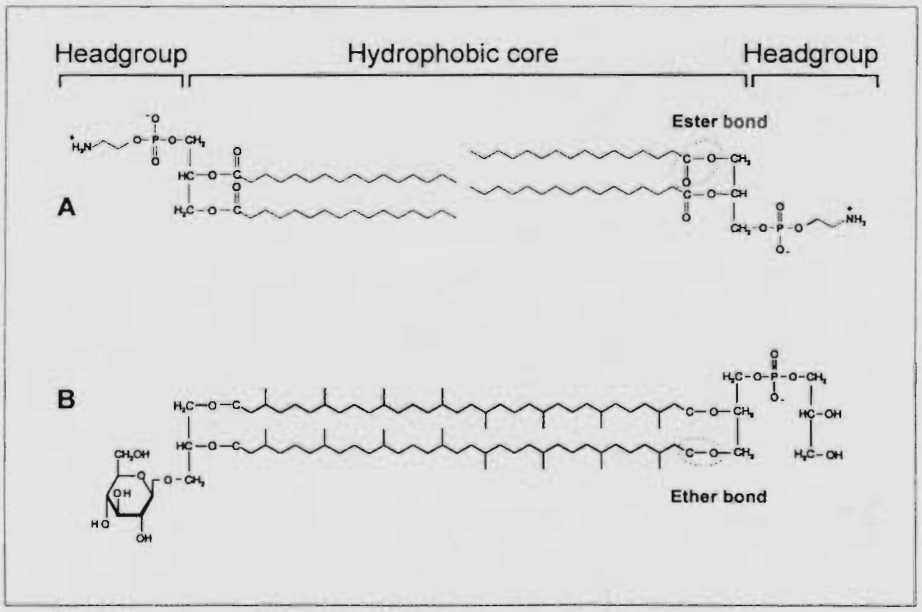

Fig.1. Lipids from Archaea and Bacteria. **A**: bilayer forming lipids in Bacteria: Phosphatidylethanolamine (PE) from *Escherichia coli*. The acyl chain is straight (not in all cases: some Bacterial lipids have a methyl branch, or a cyclohexyl group, at the end of the acyl chain, other lipids have one or more unsaturated bonds). The connection of the acyl chain with the headgroup is an ester. **B**: Monolayer forming lipids in thermo-acidophilic Archaea: Main glycophospholipid (MPL) of *Thermoplasma acidophilum*. The phytanyl chain contains isoprane-like branches. The connection of the phytanyl chain with the headgroup is an ether. Some acidophilic tetraethers contain cyclopentane rings.

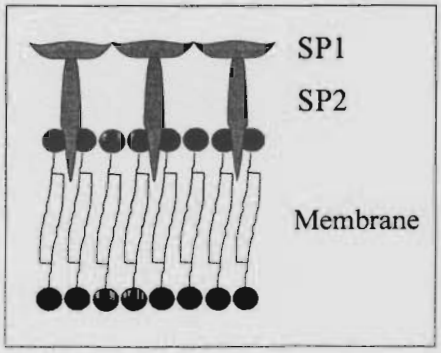

Fig. 2. Model of the membrane of *S. solfataricus*. Lipids shown here are membrane-spanning tetraether lipids.

We are especially interested in the transport proteins of *S. solfataricus*. These proteins have to face a large pH difference and have to be stable at high temperature. Such transport proteins may be good candidates for crystallization and structure elucidation.

2. Approaches

Two different approaches were used. In the first genetic approach attempts were made to express two ORF's, which are coding for putative secondary transporters. Up to now heterologous expression of these proteins in *E. coli* or *L. lactis* was not successful. At this moment this approach suffers from the lack of transformation or endogenous expression systems in *Sulfolobus* itself. Some vector systems, which have been published, were found to be useful only for some cytoplasmic proteins [5].

The second approach was the isolation of membrane vesicles from *S. solfataricus*. The isolation of such closed membrane vesicles from *S. solfataricus* membranes was hampered by the presence of the S-layer, which surrounds these cells. The S-layer consists of two proteins, SP1 and SP2 (Fig.2). SP1 can easily be stripped off, but SP2 sticks into the membranes [12]. SP2 likely prohibits the formation of closed membrane vesicles. Although the isolated membranes could not be used for uptake studies, since no electrochemical proton gradients could be generated across these membranes, the membranes were found to bind glucose.

3. Results

Glucose binding was also detected in Triton X- 100 total membrane protein extracts of *S. solfataricus*. It was concluded that these membranes contain a glucose-binding protein (GBP). GBP is tightly associated with the membrane, because it resists treatment with 22 mM Na_2CO_3, pH 10, and 1% Triton X- 100 is needed to solubilize it. The glucose-binding activity appeared to be associated with a 65 kDa protein band on SDS-PAGE, which gave a positive signal with glycoprotein stain. An affinity chromatography column, Con A, is able to bind glycoproteins and can be used for the first purification step. This step reduces the amount of proteins from a total membrane protein extract to 7-10 proteins, from which GBP can subsequently be separated by anion exchange chromatography. The glycoprotein-fraction also contained sugar-binding proteins for sucrose, maltose, cellobiose and arabinose. The isolated glucose-binding protein exhibited the same pH optimum for glucose binding as was found with crude membranes or solubilized membrane proteins. The optimal pH value was around 1.5, but significant binding activity could even be detected at a pH of 0.6.

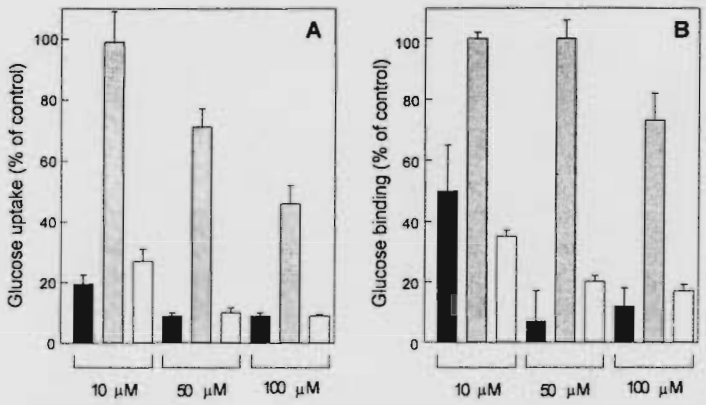

Fig. 3. Glucose uptake by *S. solfataricus* cells (A) and binding of glucose to purified GBP (B) in the presence of competing substrates. Uptake and binding assays were performed at 60°C and 1 µM [^{14}C]glucose in the presence of the indicated concentration of mannose (black), 2-deoxyglucose (grey), and galactose (white).

The K_D for glucose binding was determined to be 430 nM at pH 2 and 60°C. The substrate specificity was tested by adding competing concentrations of non-labelled sugar prior to the binding assay. Binding of glucose to the purified binding protein was strongly inhibited by galactose and mannose, but not by 2-deoxyglucose. The same substrate specificity was observed for glucose transport in intact cells (Fig. 3), indicating that GBP is directly involved in glucose uptake [1].

To identify the gene coding for GBP, the amino-terminal amino acid sequence of the purified protein was determined. A stretch of 31 amino acid residues could be determined and allowed the identification of the complete open reading frame in the genomic database of *S. solfataricus* P2. This gene codes for a protein with a molecular mass of 61 kDa. The size difference with the purified protein (~65 kDa) is most likely due to the glycosylation of the mature protein. The protein contains eleven possible glycosylation sites, while hydropathy analysis revealed strong hydrophobic regions at the amino-terminus and at the carboxyl-terminus of the protein. Both may form a transmembrane segment that anchors the protein to the cytoplasmic membrane.

The amino-terminal amino acid sequence of the purified glucose-binding protein completely matches the predicted sequence from the DNA database, except that the first 12 amino acids are lacking in the purified protein (Fig. 4). One possibility is that the protein is truncated as a result of proteolytic degradation. The other possibility is that the protein bears a signal sequence, which is processed after synthesis and before transport over the cytoplasmic membrane.

Comparisons with known signal sequences showed that archaeal flagellins have very similar characteristics in their N-terminal sequences. Kalmakoff and Jarrel [16] have shown that signal sequences of archaeal flagellins are not homologous to bacterial flagellins, but rather resemble the bacterial proteins of the IV pilin superfamily which includes also DNA uptake and protein excretion systems [13]. The genome of *S. solfataricus* bears a homologue of VirB, a component of a DNA uptake system in *Agrobacterium*. The open reading frames located.downstream of *Methanococcus voltae* flagellins show significant homology to pilB [4], a nucleotide binding protein involved in pilus function in *E. coli* [22]. This demonstrates that type IV secretion systems are also present in Archaea [2].

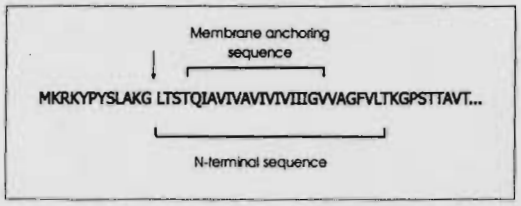

Fig. 4. Amino acid sequence of the N-terminus of GBP. The sequenced 31 amino acids and the putative membrane spanning part are indicated. The signal sequence is cleaved off at the G12. The processing site is indicated by an arrow.

Databank searches revealed that GBP shows the highest homology with the product of the *P. horikoshii* PH1214 gene (24 % identity and 40 % similarity). Downstream of PH1214 of *P. horikoshii*, two other ORFs are located in the same direction of transcription, PH1215 and PH1216 (Fig. 5). These two ORFs code for integral membrane proteins that are homologous to sugar permeases bearing the inner membrane component signature typical for binding-protein-dependent-transport systems. Further analysis of the DNA sequence surrounding the glucose-binding protein gene of *S. solfataricus* revealed three upstream genes, i.e., ORF32, ORF34 and ORF35 that are transcribed in the reverse direction (Fig. 5). ORF34 and ORF35 show homology to binding-protein-dependent sugar permeases, while ORF32 is similar to several ATP-binding proteins. Furthermore, ORF34 and PH1215 (28 % identity, 51 % similarity), and ORF35 and PH1216 (24 % identity, 54 % similarity) are homologous (Fig. 5). This genetic organisation suggests that GBP is a subunit of an ABC-transporter.

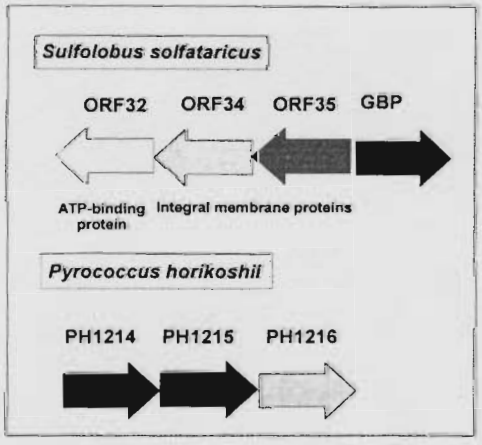

Fig. 5. Organisation of the genomic region around the glucose-binding protein (GBP) of *S. solfataricus* P2 and its homologue, PH1214, of *Pyrococcus horikoshii* OT3. Homologous genes are indicated by the same shading pattern. ORF34, ORF35, PH1215, and PH1216 code for putative membrane proteins, and ORF32 encodes an ATP-binding protein belonging to the ABC superfamily.

Trials for heterologous expression of GBP in *E. coli* have been unsuccessful. This could be due to the hydrophobic stretches, which could integrate into and distort the host membranes. Also the external pH of *E. coli* medium is higher than of *S. solfataricus*. This higher pH could prevent proper folding of GBP. On the other hand ORF32, the ATP-binding protein of the ABC-transporter, can be expressed in the cytoplasm of *E. coli*. This expression can be enhanced strongly by the co-expression of plasmid encoded tRNAs, which are rare for *E. coli*, but frequently used by *S. solfataricus*. *S. solfataricus* has a low G/C content (38%). It prefers codons which are A/T rich and in the cases of arginine, lysine and isoleucine are barely used by *E. coli*. The gene encoding the ATP-binding protein contains 42 of these codons and upon expression of this protein these tRNAs are depleted in *E. coli* resulting in low expression levels.

The ATP-binding protein was expressed with an N-terminal 6 histidine-tag and a Ni-NTA affinity chromatography column was used as a first purification step. Impurities were further removed by cation exchange chromatography. The protein, which has a molecular weight of 41 kDa, hydrolyzes ATP as shown by the release of phosphate. The optimal temperature for ATP hydrolysis is 70°C (Fig. 6).

4. Discussion

Transport of glucose in *S. solfataricus* is mediated by a high affinity binding-protein-dependent system that is specific for glucose, galactose, and mannose [1]. No 2-deoxyglucose binding was found to the membrane vesicles and to the purified binding protein. This suggests that a hydroxyl group at the C2 position of the sugar is critical for binding of the substrate, but GBP does not discriminate between C2 and C4-epimers of glucose.

A short amino-terminal sequence of 12 amino acids predicted on basis of the nucleotide-sequence is not present in the purified GBP. Similar observations have been made for the flagellin proteins in methanogens. This suggests that these proteins are processed and secreted in a similar way [2].

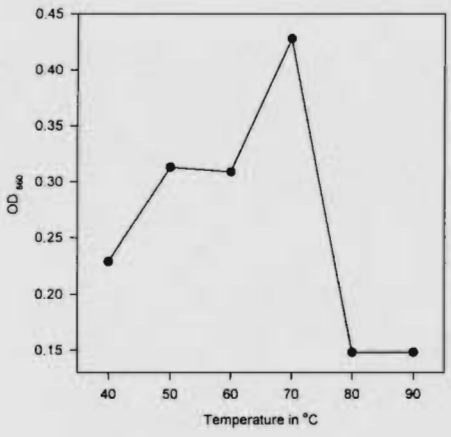

Fig. 6. Temperature optimum of ATP hydrolysis by recombinant ORF32. ATP hydrolysis is shown as release of free phosphate, which is measured by a colorimetric assay using malachite green at a wavelenght of 660 nm.

Another unusual aspect of the glucose-binding protein is its extreme acid resistance. The protein exhibits a very low pH optimum, i.e., around pH 1.5, which is comparable to that of pepsin in the stomach [11]. In this respect, GBP differs from two other extracellular enzymes of *S. solfataricus* that have been analysed, i.e., α-glucosidase and an esterase, which exhibit pH optima of 4.5 and 6, respectively [14, 20]. The purified GBP in detergent solution appeared somewhat more acid susceptible than the membrane-bound enzyme.

In Gram-negative bacteria, binding proteins exist in a soluble form in the periplasm. *S. solfataricus* lacks an outer membrane, and is instead surrounded by an S-layer. This hexagonal-paracrystalline-proteinaceous structure contains large pores of 4-5 nm to allow contact with the external medium [17]. It is thought that this structure serves as a molecular sieve, but it is not known if it can act as a barrier for proteins that are present in the space between the cytoplasmic membrane and the S-layer. The transmembrane segment(s) of GBP most likely serves as an anchor to the membrane like the lipid moiety that retains binding proteins at the cytoplasmic membrane of Gram-positive bacteria. Although the exact membrane topology of the protein is not yet known, the major part of the protein is most likely located at the outer surface of the membrane where it is glycosylated and where it can perform its function as a binding protein.

A transporter with affinity for glucose and galactose has previously been identified in *Brucella abortus* [10]. This system presumably catalyzes a sugar/H^+ symport reaction. Energetically such a mechanism would also be attractive for *S. solfataricus*, since it maintains a very large pmf across its membrane. Uptake of glucose by *S. solfataricus* mediated by a binding protein that is associated with a secondary transport system, is unlikely (15; 21), because the gene encoding the glucose-binding protein is located adjacent to genes encoding two integral membrane proteins and one ATPase subunit that are typical for ABC transporters. These three genes are transcribed in the reverse direction relative to the binding protein and a direct link is not immediately obvious. However, homologues of these genes in the genome of *Pyrococcus horikoshii* OT3 are contained in a single operon-like structure. Moreover, the two integral membrane proteins, ORF34 and ORF35, are also homologous to many other sugar permeases belonging to an ABC transporter. We therefore conclude that the glucose-binding protein is a subunit of an ABC transport system. The binding-protein-dependent maltose/trehalose transporters of *Thermococcus litoralis* [27] and *S. shibatae* [28], and the glucose transporter of *S. solfataricus*, exhibit very high affinities for their sugar substrates, i.e., in the submicromolar range. Moreover, *S. solfataricus* also contains high affinity binding proteins for at least maltose, arabinose, cellobiose and sucrose. For most of these substrates some bacteria can use phospho*enol*pyruvate dependent phosphotransferase systems (PTS). PTS has not yet been found in Archaea. Evidently, Archaea make use of ABC-transporters which activity depends on ATP levels inside of the cell. In contrast to PTS, ABC-transporter involve high-affinity-binding proteins. The high affinity of the binding proteins allows archaeal cells to efficiently utilise carbon sources in substrate poor environments such as the hydrothermal vents in the deep sea or the hot sulfuric pools.

Acknowledgements

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